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Mos Mediates the Mitotic Activation of p42 MAPK in *Xenopus* Egg Extracts

Jianbo Yue[1,*] and James E. Ferrell, Jr.[1]
[1]Department of Molecular Pharmacology
Stanford University
Stanford, California 94305-5174

Summary

The ERK1/ERK2 MAP kinases (MAPKs) are transiently activated during mitosis, and MAPK activation has been implicated in the spindle assembly checkpoint and in establishing the timing of an unperturbed mitosis [1–4]. The MAPK activator MEK1 is required for mitotic activation of p42 MAPK in *Xenopus* egg extracts; however, the identity of the kinase that activates MEK1 is unknown. Here we have partially purified a Cdc2-cyclin B-induced MEK-activating protein kinase from mitotic *Xenopus* egg extracts and identified it as the Mos protooncoprotein, a MAP kinase kinase kinase present at low levels in mitotic egg extracts, early embryos, and somatic cells. Immunodepletion of Mos from interphase egg extracts was found to abolish Δ90 cyclin B-Cdc2-stimulated p42 MAPK activation. In contrast, immunodepletion of Raf-1 and B-Raf, two other MEK-activating kinases present in *Xenopus* egg extracts, had little effect on cyclin-stimulated p42 MAPK activation. Immunodepletion of Mos also abolished the transient activation of p42 MAPK in cycling egg extracts. Taken together, these data demonstrate that Mos is responsible for the mitotic activation of the p42 MAPK pathway in *Xenopus* egg extracts.

Results and Discussion

We chose *Xenopus* egg extracts as a source for purification of the mitotic activator of mitogen-activated or extracellular signal-regulated protein kinase1 (MEK1) and p42 mitogen-activated protein kinase (MAPK) for several reasons. First, egg extracts can be driven into a permanent mitotic state by the addition of nondegradable cyclins, which allows MEK1 and p42 MAPK to be activated fully (Figure 1A; see also [1, 10]). Second, there is evidence for a role for p42 MAPK in mitosis in *Xenopus* extracts; abrogating p42 MAPK activation accelerates mitotic exit and interferes with the spindle assembly checkpoint [1, 3–5]. Also, although there is circumstantial evidence for a similar role in somatic cells—active phospho-MAPK can be detected at the spindle poles and kinetochores during mitosis, and the kinetochore phospho-MAPK staining disappears at the metaphase/anaphase transition [6, 7]—functional studies have yielded inconsistent results [8, 9].

*Correspondence: jyue@stanford.edu

Activation of MEK1 in Δ90-Cyclin B-Treated Extracts

The addition of Δ90-cyclin B also brings about the phosphorylation and activation of MEK1, as assessed by immunoblotting with phospho-MEK antibodies (Figure 1A) and by immune complex kinase assays (data not shown). Similar results have been obtained for cyclin A-treated oocyte extracts [11]. Previous work has shown that MEK1 depletion blocks Cdc2-cyclin B-induced activation of p42 MAPK, and adding back recombinant, active MEK to the MEK-depleted extract restores activation of p42 MAPK [1]. Thus, MEK1 is an essential intermediary between Cdc2-cyclin B activation and p42 MAPK activation.

Activation of a MEK-Activating Kinase in Δ90-Cyclin B-Treated Extracts

The increase in MEK activity in Δ90-cyclin B-treated extracts implies that Cdc2-cyclin B must increase the rate of MEK phosphorylation, decrease the rate of MEK dephosphorylation, or both. To check the first possibility, we added nonphosphorylated, recombinant glutathione S-transferase (GST)-MEK1-Flag to various concentrations of interphase extract or Δ90-cyclin B-treated extract and measured the initial rate of GST-MEK1-Flag phosphorylation by phospho-MEK immunoblotting. As shown in Figures 1B and 1C, there was an approximate 2–3-fold increase in the rate of GST-MEK1-Flag phosphorylation in Δ90-cyclin B-treated extracts at all extract concentrations. Similar results were obtained with a linked MEK1/p42 MAPK/myelin basic protein kinase assay (data not shown).

To check whether MEK dephosphorylation is regulated by Cdc2, we phosphorylated GST-MEK1-Flag with immunoprecipitated *Xenopus* B-Raf and [γ-^{32}P] adenosine triphosphate (ATP), added the ^{32}P-labeled GST-MEK1-Flag to interphase or Δ90-cyclin B-treated extracts, and measured the rate of disappearance of radiolabel from GST-MEK1-Flag. As shown in Figures 1D and 1E, there was no measurable difference in the rate of GST-MEK1-Flag dephosphorylation in the two types of extracts. These data suggest that the MEK phosphatases are not regulated during mitosis.

Partial Purification of a Mitotic MEK-Activating Kinase

To identify the Δ90-cyclin B-activated MEK activator, we prepared cycloheximide-treated interphase extracts and cycloheximide- and Δ90-cyclin B-treated M phase egg extracts, fractionated them by Q-Sepharose anion exchange chromatography, and assessed the MEK-kinase (MEKK) activity of each fraction. Two peaks of MEKK activity were present in the M phase extracts: one that eluted at low salt (100–150 mM NaCl, peak I) and one that eluted at higher salt (200–300 mM NaCl, peak II) (Figure 2A). Peak I was only detectable in M phase extracts, whereas peak II was present in both interphase and M phase extracts (Figure 2A). This sug-

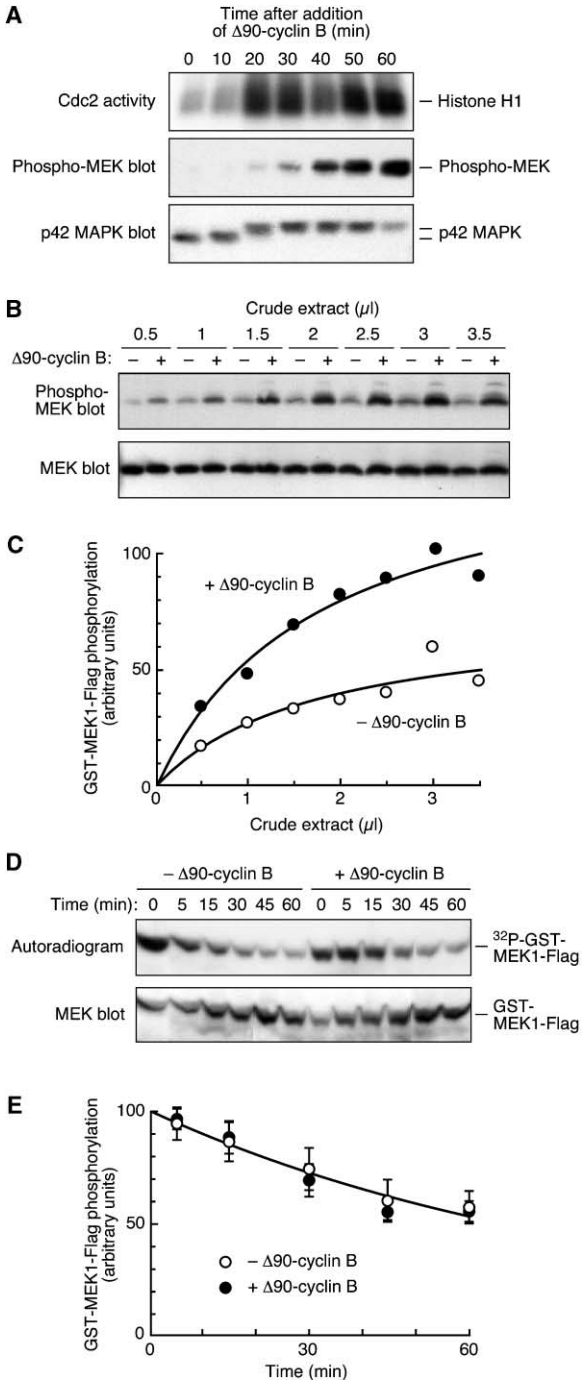

Figure 1. Activation of a MEKK Activity in Δ90-Cyclin B-Treated *Xenopus* Egg Extracts

(A) Time course of Cdc2 activation, MEK phosphorylation, and p42 MAPK phosphorylation in Δ90-cyclin B-treated extracts. Cdc2 activity was assessed by histone H1 kinase assay and autoradiography. MEK phosphorylation was assessed by phospho-MEK immunoblotting. p42 MAPK phosphorylation was assessed by immunoblotting. The electrophoretically retarded MAPK band represents phosphorylated p42 MAPK.

(B) Activation of a MEKK activity in Δ90-cyclin B-treated extracts. Various volumes of extract were treated with or without Δ90-cyclin B for 60 min. Extracts were then incubated with recombinant GST-MEK1-Flag for 10 min. The phosphorylation of GST-MEK1-Flag was then assessed by phospho-MEK immunoblotting and densitometry. Equal loading of GST-MEK1-Flag was verified by MEK immunoblotting. (C) Quantitation of the data shown in (B).

(D) Dephosphorylation of ^{32}P-labeled GST-MEK1-Flag in interphase and Δ90-cyclin B-treated extracts.

(E) Cumulative data from five dephosphorylation experiments. Points represent means ± standard error.

gested that peak I was likely to represent the activity that linked Cdc2 activation to MEK activation during M phase.

We then set out to partially purify peak I. Three purification steps (Q-Sepharose chromatography, ammonium sulfate precipitation, and Superose 6 gel filtration chromatography) afforded good purification (185-fold) and good yields (~80%) (see Table S1 in the Supplemental Data available with this article online). Moreover, the peak I activity gel filtered with an apparent molecular mass of approximately 25–45 kDa (Figure 2B). This eliminated Raf-1, A-Raf, B-Raf, and MEKK1-4 as viable candidates for the mitotic MEKK (they are larger) and raised the possibility that a monomeric form of the 39 kDa Mos protein might be the Δ90-cyclin B-activated MEK activator. As shown in Figure 2B, Mos was detectable in Superose 6 fractions 13 and 14, the same fractions that possessed MEKK activity. We therefore subjected these fractions to immunoprecipitation with either a Mos antibody or a control immunoglobulin G (IgG). As shown in Figure 2C, the Mos antibody quantitatively depleted the fractions of their MEKK activity, whereas the control IgG did not (Figure 2C); this indicated that most or all of this partially purified MEKK activity was attributable to Mos.

We also partially purified the peak II MEKK activity and demonstrated that it represents B-Raf. Our characterization of *Xenopus* B-Raf will be presented elsewhere (J.Y. and J.E.F., unpublished data).

Are the Mos Concentrations that Are Present in Interphase Extracts Functionally Significant?

Mos is present at a concentration of approximately 10 nM in mature *Xenopus* oocytes and *Xenopus* eggs [12], and after fertilization most of the Mos is degraded [13–15] (Figure 3A). Similar results are seen in electrically activated eggs and in ionophore-activated eggs (data not shown). To quantify the levels of Mos present in interphase extracts, we added ^{35}S-labeled, in vitro-translated kinase-minus Mos (without an N-terminal tag) to cycling extracts prepared from ionophore-treated eggs. Mos was degraded with a half-life of 39 min (Figure 3B), in reasonable agreement with the less quantitative estimates from Mos immunoblotting of lysates from fertilized eggs (Figure 3A). This means that during the time window of 60–90 min postactivation or postfertilization, approximately 2–3 nM Mos should remain. Similar levels of residual Mos can be seen in the published work of others [13]. This residual Mos protein could, in principle, be responsible for the transitory mitotic activation of p42 MAPK seen in dividing embryos (Figure 3A) and in cycling *Xenopus* egg extracts (Figures 3B and 4C) and for the sustained activation of p42 MAPK seen in Δ90-cyclin B-treated interphase extracts (Figure 1A).

To determine whether Mos is the main Δ90-cyclin

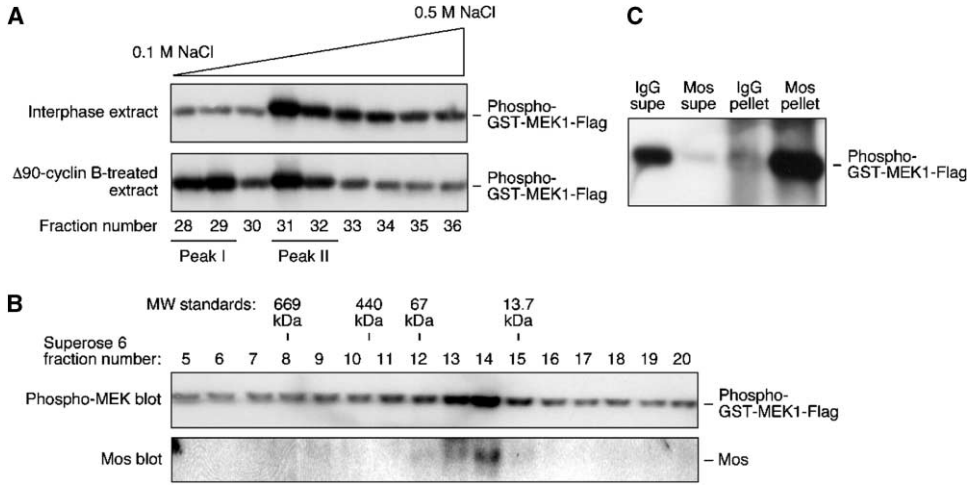

Figure 2. Fractionation of MEKK Activities

(A) Q-Sepharose chromatography. Interphase and M phase egg extracts were fractioned by Q-Sepharose chromatography. Aliquots of each fraction were incubated with recombinant GST-MEK1-Flag. Phosphorylated GST-MEK1-Flag was detected by phospho-MEK immunoblotting. Two peaks of activity were seen in the M phase Δ90-cyclin B-treated extracts.

(B) Gel filtration chromatography. MEKK activity and Mos protein coeluted with an apparent molecular weight of approximately 40 kDa.

(C) Immunoprecipitation of the partially purified MEKK activity with Mos antibodies. Aliquots (50 μl) of Superose 6 fraction 14 (panel B) were subjected to immunoprecipitation with Mos antibodies or control IgG. Portions of the resulting supernatants (supe) and pellets were incubated with GST-MEK1-Flag and MgATP, then immunoblotted with phospho-MEK antibodies.

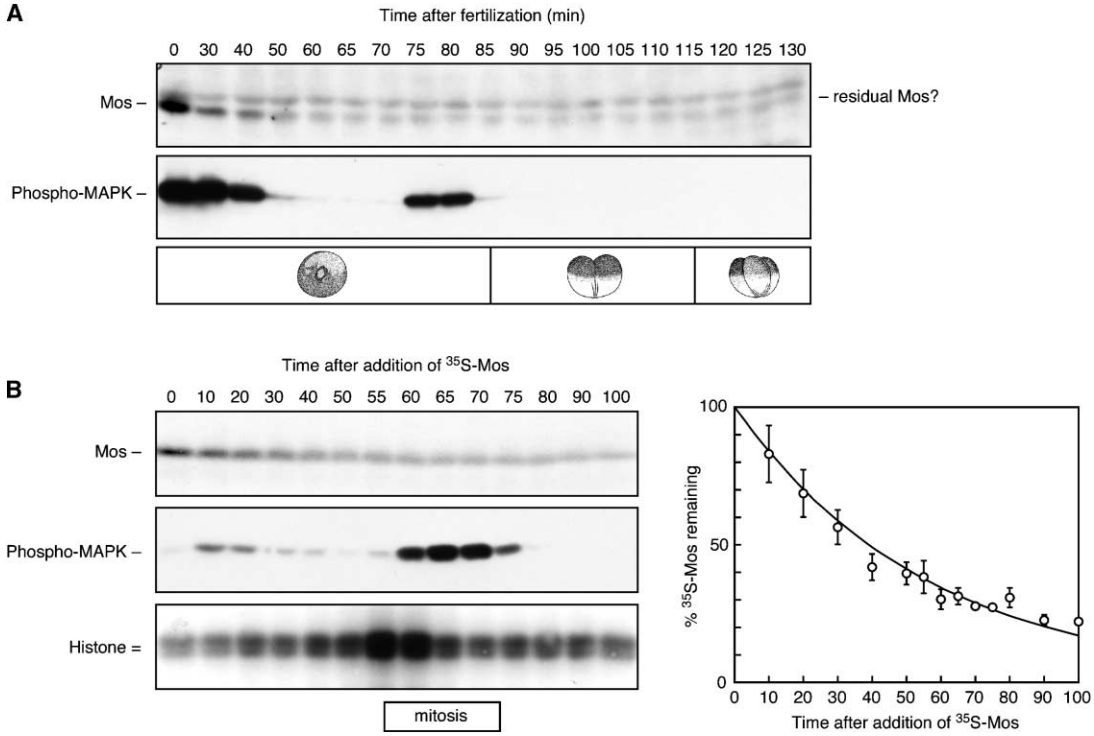

Figure 3. Mos Degradation after Fertilization and in Cycling Egg Extracts

(A) Degradation after fertilization. Eggs were fertilized in vitro, dejellied, and subjected to immunoblotting for Mos (top) and phospho-MAPK (bottom). Five embryos (Mos) or two embryos (phospho-MAPK) were loaded per gel lane. Cleavages occurred at 85 and 115 min. Embryo drawings are adapted from [27].

(B) Degradation in cycling egg extracts. Extracts were prepared after eggs were treated for 2 min with ionophore A23187 (0.8 μM). At time zero, ^{35}S-labeled in vitro-translated Mos was added. Taking aliquots every 5–10 min allowed assessment of residual Mos (by PhosphorImaging), p42 MAPK phosphorylation (by phospho-MAPK immunoblotting), and histone H1 kinase activity. Sperm morphology was used for monitoring cell cycle progression. Nuclear envelope breakdown was taken as the start of mitosis, and nuclear envelope reformation was taken as the end of mitosis. The panel on the right shows cumulative data from three independent experiments. Points represent means ± standard error.

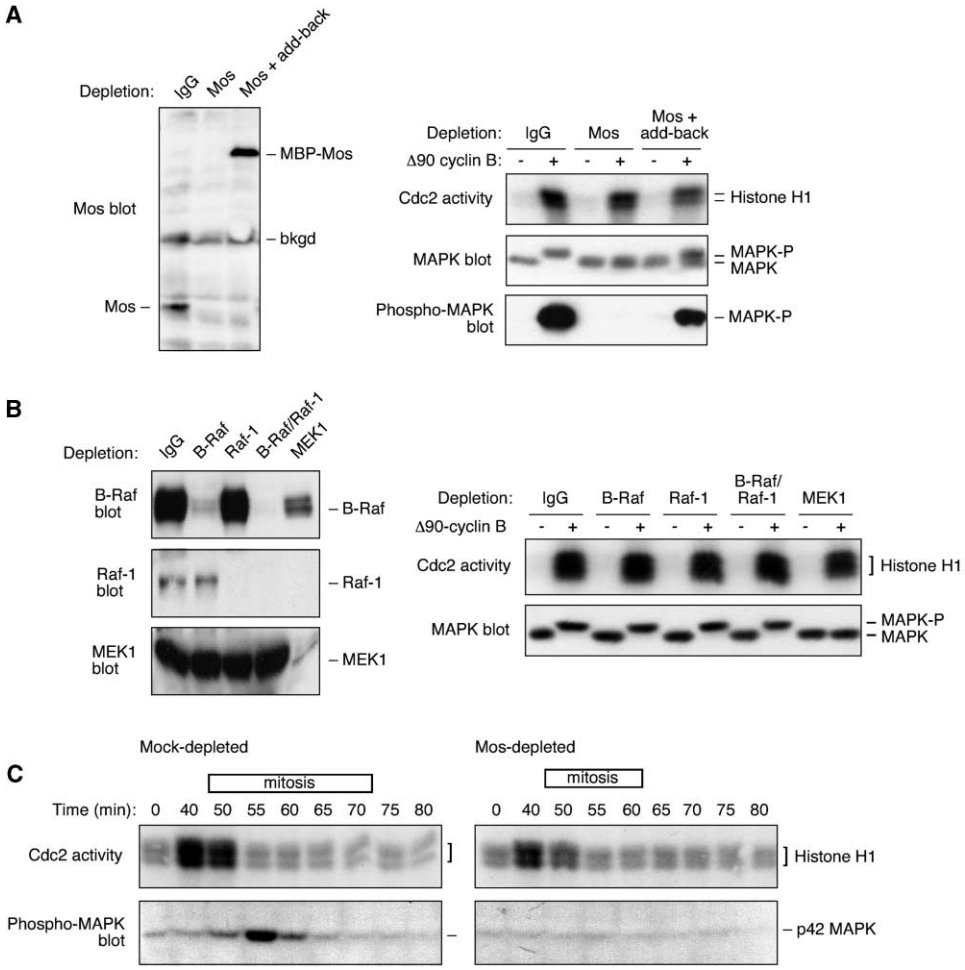

Figure 4. Depletion of Mos from Crude *Xenopus* Egg Extracts

(A) p42 MAPK phosphorylation in control and Δ90-cyclin B-stimulated extracts that were mock depleted, depleted of Mos, or depleted of Mos and supplemented with bacterially expressed MBP-Mos (10 nM).

(B) Depletion of B-Raf and Raf-1 does not block p42 MAPK activation.

(C) Mos depletion blocks the transient p42 MAPK activation that occurs in cycling *Xenopus* egg extracts. Mitotic entry and exit were assessed from sperm morphology, as described for Figure 3B.

B-activated MEKK in *Xenopus* egg extracts, we subjected crude interphase extracts to immunodepletion with Mos antibodies, and we asked whether Δ90-cyclin could still bring about p42 MAPK activation. As shown in Figure 4A, depletion of Mos completely eliminated the ability of Δ90-cyclin B to activate p42 MAPK. Adding back recombinant, bacterially expressed maltose-binding protein (MBP)-Mos partially restored the p42 MAPK response, although the amount of MBP-Mos required for restoration (~10 nM) was higher than the concentration of Mos normally present in interphase extracts (~2–3 nM) (Figure 4A). We also immunodepleted Raf-1 and B-Raf, two other MEKKs that are present in *Xenopus* egg extracts, and examined the response of p42 MAPK to Δ90-cyclin B. We found little effect of Raf-1 depletion, B-Raf depletion, or Raf-1/B-Raf double depletion on the ability of Δ90-cyclin B to bring about p42 MAPK activation (Figure 4B). In contrast, MEK1 depletion completely blocked p42 MAPK activation (Figure 4B). Thus, Mos appears to be the main MEKK involved in the mitotic activation of MEK1 and p42 MAPK.

In addition, we assessed whether Mos is responsible for the transient activation of p42 MAPK in cycling extracts. As shown in Figure 4C, depletion of Mos completely abolished mitotic p42 MAPK activation. Thus, Mos is critical both for the sustained activation of p42 MAPK seen in Δ90-cyclin B-treated extracts and for the transient mitotic activation of p42 MAPK in cycling extracts (see also Figure S1).

Concluding Remarks

The protooncoprotein Mos has long been recognized as essential for the activation of MEK1 and p42 MAPK during oocyte maturation [16–21]. Here we have shown that Mos is also the main mitotic activator of MEK1 and p42 MAPK in *Xenopus* egg extracts. At least two other MEK activators—Raf-1 and B-Raf—are present in *Xenopus* egg extracts, but both of these proteins are dispensable for Δ90-cyclin B-induced activation of p42 MAPK (Figure 4B).

Reuter and colleagues previously reported the bio-

chemical fractionation of MEKK activities from mitogen-stimulated NIH3T3 cells [22]. They found two peaks of mitogen-stimulated activity on Mono Q chromatography. One peak was identified as B-Raf. The other peak gel filtered at approximately 40 kDa, consistent with the molecular weight of Mos, but could not be immunoprecipitated with Mos antibodies. In view of the present findings, it would be interesting to reopen the question of the identity of this ~40 kDa MEKK activity.

So far, most of the work on Mos function has focused on meiosis because during meiosis the Mos protein is expressed at relatively high levels. The most obvious defect in Mos knockout mice is a diminished ability of their oocytes to arrest properly in meiosis II [16, 17, 23]; this phenotype is consistent with the idea that Mos functions primarily or exclusively during oocyte meiosis. However, the present results suggest that the low levels of Mos protein that remain after fertilization might be functionally significant as well; without this Mos, *Xenopus* egg extracts fail to activate their p42 MAPK during mitosis. This raises the possibility that low concentrations of Mos might contribute to the transient activation of MAPK and MEK seen at the spindle poles and kinetochores of somatic cells during mitosis [6, 7]. In a manner consistent with this idea, Wang et al. showed that microinjected Mos localizes to kinetochores in fibroblasts [24]. If, as seems plausible, endogenous Mos also localizes to kinetochores, it would put Mos in the appropriate location to be responsible for the mitotic activation of kinetochore-associated MEK and ERKs in somatic cells. Mos mRNA and protein [25, 26] have also been reported to be present in other somatic cells, albeit at low levels. It will be of interest to reopen the question of whether Mos is involved in M phase regulation in somatic cells as well as in oocytes and embryos.

Supplemental Data

Additional data and detailed Experimental Procedures used in this work are available at http://www.current-biology.com/cgi/content/full/14/17/1581/DC1/.

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